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1. Your reference FRENCH/GL/99/001/ABH

2. Patent application number
(The **9904804.3** -2 MAR 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

KING'S COLLEGE LONDON,
AN INSTITUTION INCORPORATED
BY ROYAL CHARTER, OF
STRAND, LONDON WC2R 2LS.

Patents ADP number *(if you know it)* 59 47494 004

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention

IDENTIFICATION OF BACTERIA

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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IDENTIFICATION OF BACTERIA

This invention relates to the identification of bacteria and more particularly, although not exclusively, to the identification of clinically important bacteria in biological samples e.g. blood. The invention is of special application to the identification of clinically important bacteria isolated in a hospital laboratory and obtained directly from clinical specimens, including positive blood culture bottles and fresh blood specimens.

Eight bacterial species account for 65% of all blood culture isolates, although this varies with patient population. Typically these are Escherichia coli (~20%), Staphylococcus aureus (~20%), Pseudomonas aeruginosa (7%), Enterococcus spp. (5%), Klebsiella spp. (~5%), Enterobacter spp. (~4%), Proteus spp, and Pneumococci (~3%). In addition coagulase negative Staphylococci are frequently isolated from patients with intra-vascular devices but many of these isolates are clinically insignificant. The remaining 35% of blood culture isolates comprise upwards of 50 different species. Rapid detection of these numerous species with a single test would be very useful.

In recent years much effort has been invested in the production of species specific primers which can be used to identify an organism in a simple PCR reaction. If a PCR product of the expected size is produced with a set of these primers the presence of the target bacterium can be predicted

with almost total certainty. Unfortunately this approach is not ideal for analyzing samples which may contain one of many pathogens. Analysis of such specimens using this approach requires a multiplex PCR containing a complex mixture of primers, a series of individual PCR reactions run in parallel to detect each species which may be present, or a series of PCR reactions run sequentially. Because of the potentially large number of different bacterial species that may be isolated from blood, these methods are unsatisfactory for the routine screening of general microbiological specimens.

A better approach is to use a single pair of primers to amplify DNA from a variety of organisms and then to analyze the sequence of the resulting product to determine from which species it originated. Primers directed at conserved stretches of DNA will produce a PCR product from almost all species of bacteria. The region usually chosen is the 16S rDNA or the 16S 23S rDNA spacer region. The 16S 23S rDNA spacer region is highly variable within many species, frequently containing tRNA genes, and the length and sequence of amplified products can be used to type strains within a single species. In contrast the 16s rDNA is highly conserved and, as a large amount of sequence data is available on public computer databases, sequence data can give a definitive identification of the species of a bacterium in many cases. Unfortunately some species of clinical significance have identical or very similar 16s rDNA sequences which would be impossible or difficult to discriminate using this region alone.

We have now found that by targeting the large ribosomal sub-unit (23s rDNA) with novel specially designed oligonucleotide primers, specified hereinafter, and amplifying a portion of this DNA we can identify a

large number of bacteria by means of a single test or at most a very small number of tests. For convenience, amplification by means of the polymerase chain reaction (PCR) will be referred to throughout the following description. It will be appreciated, however, that any other amplification technique can alternatively be used e.g. transcription mediated amplification (TMA), reverse transcriptase polymerase chain reaction (RTPCR), Q-beta replicase amplification, and single strand displacement amplification. Some modification of the primers used for PCR may be necessary when using these alternative methods.

In accordance with the present invention the bacterial species are detected by amplifying bacterial 23S rDNA, and identified by using the amplified product (amplicon) to probe one or more oligonucleotides in a reverse hybridization system. After amplification by universal primers, the sequence of the amplicon has to be determined. Direct sequencing is complex and expensive. Sequence variation can be identified by restriction digests, but this is not a practical way to detect a wide range of variants. According to this invention the labeled PCR product is preferably hybridized to a panel or an array of oligonucleotides immobilized on a solid phase such as, for example, nylon membranes or synthesized in situ on silicon wafers. Since both the target and the probe are present at much higher concentrations than is typical for a Southern blot these hybridization reactions can be carried out in very short periods of time (less than 1 hour). This method is referred to as reverse hybridization. Reverse hybridization allows an almost infinite series of sequence variations to be positively identified and lends itself to automation.

The present invention comprises primers that amplify a portion of the 23S rDNA. The DNA sequences of these primers is set out below. The PCR products produced by these primers, from a range of medically important Gram positive and Gram negative bacterial cultures, were characterized by hybridization to an array of oligonucleotides designed to identify taxonomic groups. Using this procedure, which took less than four hours, we have been able to identify a wide range of genera and species. This approach potentially allows bacteria and mixtures of bacteria to be identified by molecular methods without the need for a priori knowledge of the causative agent or agents.

The oligonucleotide probes can be used singly for the identification of certain individual species or in a panel or array for the identification of many different species. There is theoretically no limit to the number of oligonucleotide targets employed and the number of species that can be identified.

Ideally the oligonucleotides used should hybridize only to one bacterial species and to all members of that species. Thus with an ideal array, a unique profile consisting of species specific spots would be seen, giving identification to the species level. In practice, two or more oligonucleotide spots may be required for many species and in some cases several such spots may allow identification of variation within a species. In addition, some identifications can be made by comparing the relative intensities of hybridization of individual species to individual oligonucleotides. The assessment of hybridization can be quantified by visual or automated methods.

For example, 27 oligonucleotides have been used for the unambiguous identification of Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus feacium and Enterococcus feacalis , as well as Staphylococcus aureus, coagulase negative Staphylococcus, Listeria species, Stenotrophomonas maltophilia , Burkholderia cepacia, and Escherichia coli.

The detection of short sequences in amplified DNA is a straightforward procedure that can be carried out on a massively parallel scale. This may be achieved by hybridizing a labeled PCR product to an array of oligonucleotides immobilized on a solid support e.g. a membrane, glass slides, or microtitre trays, or synthesized in situ on silicon wafers. This assay can be easily extended to identify a wider range of bacterial species with the addition of oligonucleotides without increasing the complexity of performing the assay.

PRIMERS AND OLIGOS

The sequences of the primers and oligonucleotides used are given below and expressed in standard IUB/IUPAC nucleic acid code. The primers are appropriately labelled e.g with Digoxigenin (as in the Example given below), biotin, or fluorescein. Any other labelling system can be used. Hybridization can also be detected by using the oligonucleotides to construct molecular beacons.

Forward primer	ST23SP6	5'gcgatttcygaaygggggraaccc
Reverse primer	ST23SP10	5'digoxigenin-ttcgcctttccctcacggtact

The Oligonucleotides are listed in Figures 1a and 1b.

METHODOLOGY

The methods we have used are described as follows:

Bacterial strains. The stored strains used are listed in Table 1. Organisms were stored in glycerol broth at -70° C.

Blood cultures. Blood cultures may be performed by using an enrichment technique e.g. the Vital® automated system (BioMerieux, France). In this method up to 10 mL blood are placed in anaerobic and aerobic Vital blood culture bottles. The bottles are then incubated in theVital machine and continuously monitored for evidence of bacterial growth. When possible growth is identified, the bottle is removed from the incubator and a sample taken for Gram staining and subculture to agar plates. Over a period of 25 days an additional sample of 100 microlitres for DNA extraction was taken from 116 unselected positive blood culture bottles, as described below. The DNA assay was performed without knowledge of the patient details or the initial Gram stain result.

Extraction of bacterial DNA from pure bacterial cultures. Stored organisms were sub-cultured onto Columbia Blood Agar plates (Oxoid, UK). A single colony of overnight growth at 37°C was suspended in 100 microlitres of distilled water containing 1 microlitre of a 1 mg/ml solution of lysostaphin (Sigma Chemical Co. UK) and incubated at 37°C for 10 minutes. The tubes were then transferred to a thermo-cycler (Perkin-Elmer 2400 Gene amp PCR system) and heated to 95°C for 10 minutes.

Finally they were spun at 13,000 rpm for 2 minutes in a micro-centrifuge and 1 ml of the supernatant used in the 23S PCR described below.

Extraction of bacterial DNA directly from Vital blood culture bottles.

DNA was extracted from all positive blood culture bottles in a Class II safety cabinet using the following protocol. Two to four drops of the broth were transferred into 0.5 ml of sterile distilled water at the time of aspiration for Gram stain and subculture. The tubes were spun at 13,000 rpm in a micro-centrifuge for 2 minutes and the supernatant discarded. The pellet was re-suspended in 100 microlitres of distilled water containing 1 microlitre of a 1 mg/ml solution of lysostaphin (Sigma, UK) and incubated at 37°C for 20 minutes in a dry block (Scotlab, UK). The temperature was then raised to 95°C and the tubes incubated for a further 15 minutes. Finally the tubes were spun at 13,000 rpm for 2 minutes in a micro-centrifuge and 1 microlitre of the supernatant used in the 23S PCR described below.

Design of primers to amplify 23S bacterial rDNA.

Forward primer ST23SP6 5' gcgatttcygaaygggggraaccc

Reverse primer ST23SP10 5'digoxigenin-ttcgcctttccctcacggtact

Primers were commercially synthesized (Amersham Pharmacia, Amersham, UK). A PCR master mix containing 1 x DnaZyme buffer (Flowgen, UK), 1 microMole Primer ST23SP6, 2 microMoles Primer SP23SP10, and 150 microMoles of each dNTP was made up in 5 ml quantities. Forty microlitre aliquots of the master mix were dispensed into 100 microlitre PCR tubes. When the DNA extracts were available 1 microlitre of the appropriate extract and 1 unit of DnaZyme DNA polymerase (Flowgen, UK) added to each tube. The PCR mixes were

then subjected to 5 cycles of 95°C for 15 seconds, 55°C for 15 seconds plus 72°C for 15 seconds, followed by 25 cycles of 95°C for 15 seconds plus 65°C for 30 seconds. The presence of a PCR product was confirmed by agarose electrophoresis of 5 microlitres and visualized with ethidium bromide.

Sequence determination of primary pathogens and identification of potential reverse hybridization targets.

Where species information was not available, we sequenced PCR products from selected isolates in our organism collection. This was supplemented by sequence data from products that failed to hybridize with the early oligonucleotide arrays or gave erroneous identifications. All the oligonucleotides chosen were targeted at sequences within a variable region of the PCR product. Using this sequence information, a panel of oligonucleotides with similar calculated melting temperatures was designed.

Production of the hybridization membranes.

The target oligonucleotides and layout are shown in Figure 1 and Figure 2. Oligonucleotides were synthesised and 50pg of each in 0.3 microlitres of water were spotted onto a specific position on a nylon membrane (MAGNA Micron Separations inc. MA, USA). A 3 mm grid was printed on the membrane with a bubble jet printer to allow the spots to be more accurately positioned. Strips were made in batches of 20. Once all the oligonucleotides had been applied the strips were dried and exposed to short wave UV in an Amplirad light box (Genetic Research Instruments, Essex, UK). The length of exposure was found to have a marked effect on the intensity of the resulting spots: with our UV illuminator 30 seconds was found to give the optimal spot intensity. After the oligonucleotides

had been cross-linked to the membrane, any unbound oligonucleotides were removed by washing twice in 0.5 x SSC plus 0.1 % SDS for 2 minutes at 37 °C. The strips were dried and stored at room temperature ready for use.

Hybridization protocol.

The digoxigenin labeled 23S rDNA amplicons were hybridized to the oligonucleotide arrays using the following protocol. Each membrane was numbered and placed in a separate 2.5 ml screw-topped micro-centrifuge tube containing 0.5 ml of 5 x SSC plus, 0.1% N-laurylsarcosine, 0.02% SDS, and 1% blocking reagent (Boehringer Mannheim, Germany). The digoxigenin PCR products were heated to 95°C in a thermal cycler and the appropriate PCR product added directly to each tube. The hybridization was continued for 45 minutes at 50°C with gentle agitation. The strips were then removed from the tubes washed four times in 25 ml 0.25 x SSC plus 0.1% SDS, for each 20 strips, at 37°C for 2 minutes. Any hybridization was detected using an anti-digoxigenin antibody conjugated to alkaline phosphatase amd detected colorimetrically (Boehringer Mannheim system). Color development was clearly visible between 15 minutes and 1 hour.

Assessment of the primers. The effectiveness of the primers was first assessed with DNA extracts from 79 stored bacterial isolates representing 28 species (Table 1). All the isolates tested produced products. A band of approximately 420 bp was produced with Gram positive bacteria and one of 390 bp for the Gram negative bacilli. Two isolates ofCandida albicans were also processed using the same protocol but no PCR products were seen. No bands were seen in the DNA negative amplification controls.

Hybridizations from enrichment broths.

Over the course of the study samples from 112 culture positive Vital bottles were subjected to PCR on the day they became positive. Examples of the strips obtained are shown schematically in Figure 2. Eighty-three bottles (74.1%) produced correct identifications. These included four (3.8%) in which mixed cultures were correctly identified. One contained Pseudomonas aeruginosa plus Enterococcus faecalis, one Pseudomonas aeruginosa plus Stenotrophomonas maltophilia, and two Staphylococcus aureus plus Enterococcus faecalis. The remaining bottles either contained no bacteria to which oligonucleotides were targeted or a PCR product was not obtained.

ASSAY PROTOCOL

SOLUTIONS NEEDED

(1) Polymerase Chain Reaction mixture:

Forward primer ST23SP6

Reverse primer ST23SP10

The PCR master mix was made up in 2.5 ml quantities containing all the ingredients for PCR except DNA polymerase. 12.5 microlitre each primer 1 microgram/microlitre (Pharmacia), 5 microlitre each dNTP 100 mM (Pharmacia), 250 microlitres 10 x DnaZyme buffer (Flowgen, Staffordshire, UK), 2.2 ml water. This mixture should then be dispensed in 45 microlitre aliquots into 200 microlitre reaction tubes and 1 unit (0.5 microlitre) of Taq polymerase (DnaZyme) added to the tubes just before they are required.

(2) Maleic acid buffer pH (7.5): 4.13 g sodium chloride and 5.53g maleic acid in 500 ml of water, pH with 5 M NaOH

(3) Detection buffer pH (9.5): 6.05g tris-base and 2.97g NaCl in 500 ml of water, pH with 10 N HCl

(4) Blocking solution: 0.1 g Boehringer Mannheim blocking solution in 5 ml of detection buffer: make 2 hours before required.

(5) SSC: (20x) 3 M NaCl plus 0.3 M sodium citrate. Dilute to 0.25 x SSC and keep at 37°C ready for use.

(6) BCIP: 50 mg/ml 5-bromo-4-cloro-3-indolyl phosphate toluidinium salt in 100% dimethylformamide

(7) NBT: 75 mg/ml nitroblue tetrazolium salt in 70% dimethylformamide

METHOD

This procedure will identify bacteria from positive Vital blood culture bottles (Bio-Merieux, France). When aspirating the broth for Gram staining and sub-culture add 2 to 4 drops of the positive Vital broth to one of the 2 ml screw-capped tubes containing 0.5 ml of sterile water and label the tube with the lab number.

DNA extraction (To be carried out in the containment level 3 laboratory)

(1) Spin the screw-capped tubes at high speed (10,000g) for 4 minutes in a sealed rotor centrifuge.

(2) In a class 1 hood open the rotor and tubes and discard the Supernatant.

(3) Add 100 microlitres of a 1 microgram/ml solution of lysostaphin (Sigma UK) made up in water.

(4) Place the tubes in a covered dry block and incubate at 37° C for 20 minutes.

(5) Turn the dry block up to 95° C and leave for 15 minutes.

The PCR and hybridization may now be carried out on the open bench in a laboratory.

Preparation of the hybridization strips

(1) Using a bubble jet printer print a grid of 20 strips onto a 18cm by 3cm section of nylon membrane (Magna nylon, MSI Westboro MA).

(2) The vertical divisions between each strip should then be cut with a scalpel to avoid bleeding of the spots between strips.

(3) Approximately 0.3 microlitres of each oligonucleotide (1 mg/ml solution in water) should then be spotted onto the appropriate position on each strip (see Figure 1).

(4) Once dry, the membrane should be cross-linked by exposing to short wave UV in the Amplirad (GRI instruments UK) for 30 seconds.

(5) The membrane should then be washed 2 times in 50 ml of 0.5 x SSC for 2 minutes and air dried.

(6) The membrane can now be stored dry at room temperature ready for use.

PCR amplification

Add 1 microlitre of the DNA extract to 45 microlitres of the PCR mixture containing 0.5 microlitres of DnyaZyme (Flowgen) in a 200 microlitre PCR tube. A PCR negative control containing no bacterial DNA must be run alongside each set of PCR reactions.
In the PE thermal 2400 cycler (Perkin-Elmer Ltd.) carry out 5 cycles of 95° for 30 sec, 55°C for 15 sec, 72^0 C for 30 sec, followed by 25 cycles of 95°C for 15 sec, 65°C for 30 sec.

Hybridization

(1) Heat the PCR reactions to 95° C in the thermal cycler for 5 minutes.
(2) Label some hybridization strips and cut out with a scalpel and place in a screw-capped tube containing 0.5 ml of hybridization solution (5 x SSC, 0.01% SDS, 0.01 % N-laurylsarcosine, 1 % blocking reagent (Boehringer Mannheim Germany)).
(3) Pipette the PCR reactions into the appropriate tubes.
(4) Hold the hybridization reactions at 50° C for 45 minutes with gentle agitation.

Detection of hybridization

(1) Wash the strips 4 times in 25 ml of 0.25 x SSC + 0.1 % SDS at 37°C for 2 minutes.
(2) Flood the strips with 5 ml of blocking solution and leave for 15 minutes.
(3) Pour off the blocking solution and replace with 5 ml of maleic acid

buffer containing 1 microlitre of the Anti-digoxigenin antibody conjugate (Boehringer Mannheim, Germany). Leave for 10 minutes.

(4) Wash the strips 4 times in 25 ml of maleic acid buffer for 1 minute.

(5) Flood the strips with detection buffer.

(6) Prepare 5 ml of the detection solution by adding 45 microlitres of BCIP and 35 microlitres of NBT to 5 ml of detection buffer.

(7) Pour off the detection buffer from the strips and replace with the detection solution prepared above.

(8) Leave the strips in the dark for 15 minutes then examine them for detectable hybridization. Record the results, after 45 minutes and terminate the development by washing the strips in distilled water.

CONCLUSION

The method of the present invention can be used to identify bacteria in settings other than those descibed above, both clinical and non-clinical, and in pure cultures after isolation. The method overcomes the problems of other similar molecular diagnostic techniques described above. It allows rapid diagnosis of such organisms in blood or blood cultures or in other clinical specimens such as cerebrospinal fluid, urine, joint fluid, swab specimens, and abscesses.. It provides a set of universal primers and experimental conditions that can be used to amplify potentially characteristic sequences of bacterial 23S rDNA. In particular, it provides a series of specific oligonucleotide targets for the identification of clinically important bacteria.

CLAIMS

1. A method for identifying bacteria in a sample which comprises amplifying a portion of the 23S rDNA present in the sample and testing the resulting amplicon by hybridisation to one or more oligonucleotides designed to identify one or more bacteria likely to be present in the sample.

2. A method according to claim 1, in which amplification is carried out by the polymerase chain reaction (PCR)

3. . A method according to claim 1, in which amplification is carried out by transcription mediated amplification (TMA).

4.. A method according to claim 1, 2, or 3, in which primers of the following sequences are used for the amplification:

Forward primer 5'GCGATTTCYGAAYGGGGRAACCC
Reverse primer 5'TTCGCCTTTCCCTCACGGTACT

5. A method according to any of claims 1 to 4, using one or more of the oligonucleotide markers listed in Figure 1a and 1b.

6. A method according to any of the preceding claims, in which the oligonucleotide markers are attached to a single test strip.

7. DNA having the sequence

5'GCGATTTCYGAAYGGGGRAACCC

8. DNA having the sequence

5'TTCGCCTTTCCCTCACGGTACT

9. A DNA sequence according to claim 7 or 8, being a labelled sequence.

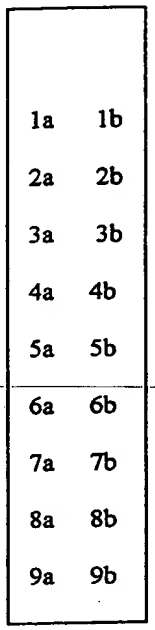
10. A Digoxigenin-labelled DNA sequence according to claim 9,

11. Oligonucleotides selected from the sequences listed in Figure 1a and 1b.

12. A diagnostic kit for the identification of bacteria comprising oligonucleotide primers as specified in claim 4.

13. A diagnostic kit for the identification of bacteria comprising oligonucleotide probes as specified in claim 11.

Figure 1a Layout of the strips and oligonucleotides used in the blood culture study.



Oligo	Target organisms	Sequence 5′ to 3′
1a	*Proteus mirabilis*	AATAGCAGTGTCAGGAGAACGGTCT
1b	*Proteus mirabilis*	ATAGCCCCGTATCTGAAGATGCT
2a	*Klebsiella oxytoca*	TCCCGTACACTAAAACGCACAGG
2b	*Klebsiella pneumoniae*	TCCCGTACACCAAAATGCACAGG
3a	*Enterobacter cloacae*	TCCCGTACACGAAAATGCACAGG
3b	*Esh.coli, Citrobacter* spp.	CCCGTACACAAAAATGCACA
4a	*Streptococcus* spp. A	AGAAGAATGATTTGGGAAGATC
4b	*Pseudomonas aeruginosa*	GCTTCATTGATTTTAGCGGAAC
5a	*Streptococcus* spp. B	AGAAGAAGACCTTGGGAAAGG
5b	*Enterococcus faecalis*	GGTAGTCTGTTAGTATAGTTGAAG
6a	*Streptococcus* spp. B	AGAAGAACTACCTGGAAGGT
6b	*Enterococcus faecium*	GGTAGTTCTTTCAGATAGTCGG
7a	*Staphylococcus aureus*	ACGGAGTTACAAAGGACGACATTA
7b	*Staphylococcus* spp.(+*Listeria* spp.)	GGTTGTAGGACACTCTATACGGAGTT
8a	*Staphylococcus epidermidis*	ACGGAGTTACAAAAGAACATGTTAG
8b	*Staphylococcus carnosus*	ATGGAGTTACAAAAGAATCGATTAG
9a	*Burkholderia cepacia*	CGTATTGTTAGCCGAACGCTCT
9b	*Stenotrophomonas maltophilia*	AGCCCTGTATCTGAAAGGGCCA

Figure 1b. Oligonucleotides for use in an extended array.

Oligo	Target organisms	Sequence 5′ to 3′
9c	*Listeria* spp.	ACGGAGTTACAAAAGAAAGTTATAA
6d	CNS A	ACGGAGTTACAAAAGAACATGTTAGTTTTT
7d	CNS B	ACGGAGTTACAAAAGAATTTGTTAGTTTTT
1c	*Esh. coli* 1	CCAGAGCCTGAATCAGTGTGT
2c	*Esh. coli* 2	CCAGAGCCTGAATCAGTATGTG
3c	*Salmonella* spp.	AGAGCCTGAATCAGCATGTGT
4c	*Heamophilus influenziae*	GTGTGGAGAATGTGTTGGGAAG
5c	*Aeromonas* spp.	TGGAACGGTCCTGGAAAGGC
6c	*Staph. warneri*	ACGGAGTTACAAAAGTCTATATTAGTTTTT
7c	*Staph. saprophiticus*	ACGGAGTTACAAAAGAACAGACTAGTTTTT
8c	*Staph. haemolyticus*	ACGGAGTTACAAAGGAATATATTAGTTTTT
10a	*Streptococcus* spp. A	AGAAGAATGATTTGGGAAGATC
10b	*Streptococcus* spp. B	AGAAGAAGACCTTGGGAAAGG
10c	*Streptococcus* spp. C	AGAAGAACTACCTGGGAAGGT
1d	*Plesiomonas shigelloides*	GTTAGTGGAACGGATTGGAA
2d	*Neisseria gonorrhoeae*	TGACCATAGCGGGTGACAGTC
3d	*Neisseria meningitidis*	TGACCATAGTGGGTGACAGTC
4d	*Campylobacter* spp.	GTGAGTTTAGCAGAACATTCTG
5d	*Campylobacter lari*	TAAGTAAGGTTAGTAGAACACTCT
6d	*Helicobacter pylori*	CATCCAAGAGAACGCTTTAGCA
7d	*Ralstonia* spp.	AATGGGATCAGCCTTGTACTCT
1e	*Esh. coli* 3	TCTGGAAAGGCGCGCGATACA
2e	*Enterobacter* 1	GTCTGGAAAGTCCGACGGTAC
3e	*Chlamydia pneumonae*	ACCATATTTTTAATATGGGG
4e	*Chlamydia psittaci*	CCACATTTTTAATGTGGGG
5e	*Chlamydia trachomatis*	CCGAGCTGAAGAAGCGAGGG
6e	*Coxiella burnetti*	CCTTTCGAGGTTATGTATACTGAA
7e	*Rhodococcus erythropolis*	GGTGTTGCATTCGTGGGGTTG
8e	*Rhodococcus fascians*	GGGTTGCGTATGGAGGGTTG
9e	*Mycobacterium tuberculosis*	GCGCTACCCGGCTGAGAGG
10e	*Mycobacterium avium*	CTACCTGGCTGAGGGGTAGTC
1f	*Mycobacterium kansasii*	GGACGATACGTCTCAGCTCTA
+ve	Positive control	AGTAGCGGCGAGCGAAACGG

Figure 2. Summary hybridization results. Numbers at the top of the strips indicate the number of strains tested. Solid circles indicate strong Hybridization and empty circles weak hybridization.

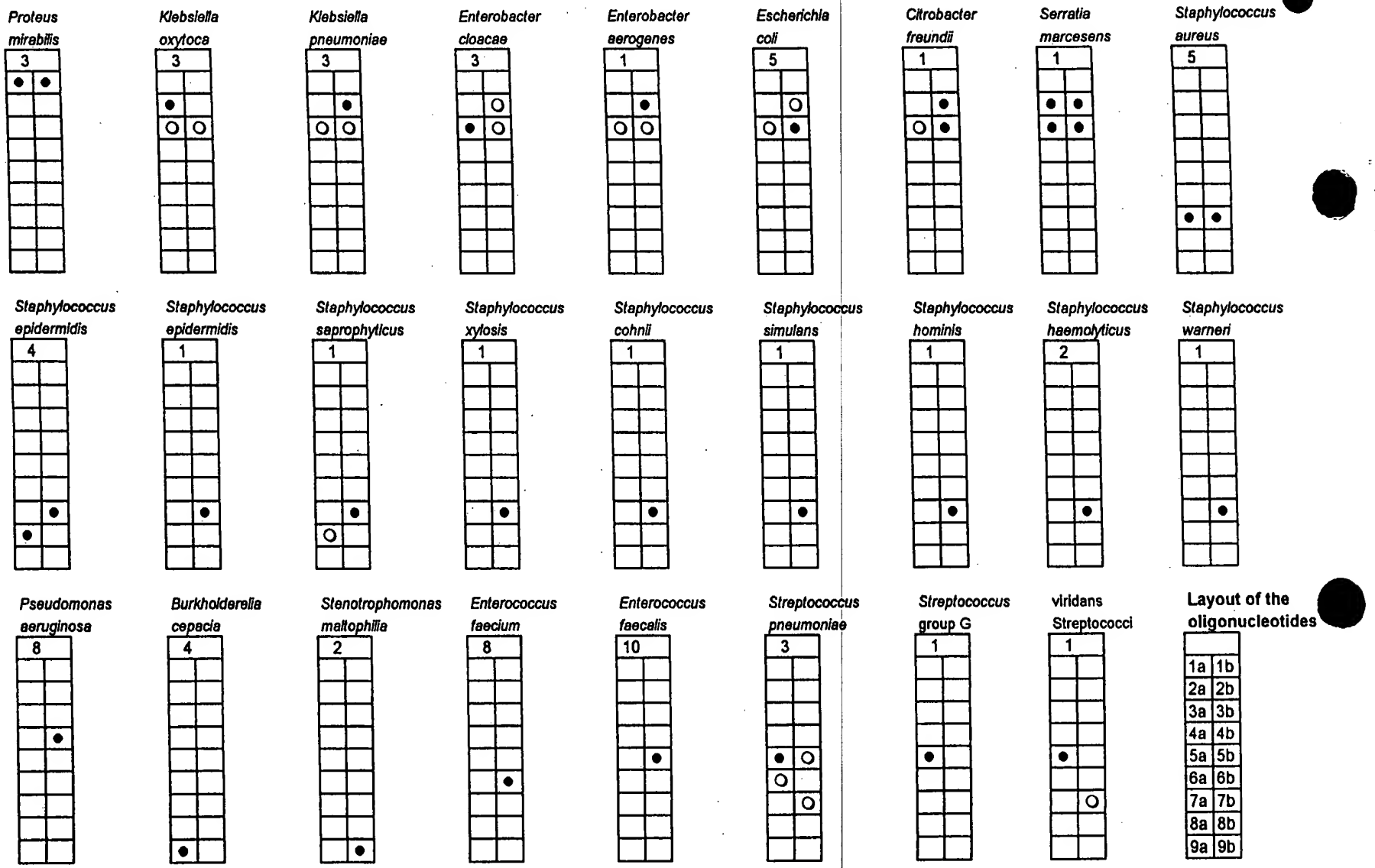


Table 1. Strains used in this study and results of PCR amplifications and hybridizations from culture.

Origin	Species	laboratory Code	Hybridization Strong	Weak
Blood culture STH	*Staphylococcus epidermidis*	36839	7b, 8a	
Blood culture STH	*Staphylococcus epidermidis*	36938	7b, 8a	
Blood culture STH	*Staphylococcus epidermidis*	44.3	7b, 8a	
Blood culture STH	*Staphylococcus epidermidis*	37061	7b, 8a	
Blood culture STH	*Staphylococcus epidermidis*	NCTC11047	7b	
Blood culture STH	*Staphylococcus warneri*	B5	7b	
Blood culture STH	*Staphylococcus saprophyticus*	B6	7b	8a
Blood culture STH	*Staphylococcus xylosus*	B7	7b	
Blood culture STH	*Staphylococcus cohnii*	B8	7b	
Blood culture STH	*Staphylococcus simulans*	B9	7b	
Blood culture STH	*Staphylococcus hominis*	B10	7b	
Blood culture STH	*Staphylococcus haemolyticus*	B11	7b	
Blood culture STH	*Staphylococcus haemolyticus*	31871	7b	
NCTC	*Staphylococcus aureus*	NCTC6571	7b 7a	
GH	*Staphylococcus aureus* (MR)	GH25	7b 7a	
GH	*Staphylococcus aureus* (MR)	GH7	7b 7a	
Blood culture STH	*Staphylococcus aureus* (MR)	816.98	7b 7a	
Blood culture STH	*Staphylococcus aureus* (MS)	36989	7b 7a	
Blood culture STH	*Streptococcus milleri*	676.98	No Hybridization	
Blood culture STH	*Streptococcus milleri*	662.98	No Hybridization	
Blood culture STH	*Streptococcus pneumoniae*	697.98	5a	5b 7b 6a
Blood culture STH	*Streptococcus pneumoniae*	76a.98	5a	7b
Blood culture STH	*Streptococcus pneumoniae*	736.98	5a	7b
Blood culture STH	*Streptococcus* spp. (viridans)	738.98	5a	7b
Blood culture STH	*Streptococcus* GroupG	776.98	5a	
feces (VRE)	*Enterococcus faecium*	BM4147	6b	
feces (VRE)	*Enterococcus faecium*	BM4152	6b	
feces STH	*Enterococcus faecium*	7	6b	
feces STH	*Enterococcus faecium*	24	6b	
feces STH	*Enterococcus faecium*	39	6b	
feces STH	*Enterococcus faecium*	40	6b	
Blood culture STH	*Enterococcus faecium*	848.98	6b	
Blood culture STH	*Enterococcus faecium*	665.98	6b	
feces STH	*Enterococcus faecalis*	20	5b	
feces STH	*Enterococcus faecalis*	23	5b	
feces STH	*Enterococcus faecalis*	24	5b	
feces STH	*Enterococcus faecalis*	25	5b	
feces STH	*Enterococcus faecalis*	27	5b	
feces STH	*Enterococcus faecalis*	82	5b	
Blood culture STH	*Enterococcus faecalis*	707.98	5b	
Blood culture STH	*Enterococcus faecalis*	706.98	5b	
Blood culture STH	*Enterococcus faecalis*	708.98	5b	
Blood culture STH	*Enterococcus faecalis*	835.98	5b	
NCTC	*Escherichia coli*	NCTC8879	3b	3a, 2b
Blood culture STH	*Escherichia coli*	817.98	3b	3a, 2b
Blood culture STH	*Escherichia coli*	794.98	3b	3a, 2b
Blood culture STH	*Escherichia coli*	829.98	3b	3a, 2b
Blood culture STH	*Escherichia coli*	780.98	3b	3a, 2b
Blood culture STH	*Klebsiella oxytoca*	800.98	2a	3a, 2b
Blood culture STH	*Klebsiella oxytoca*	243a.95	2a	3a, 2b
Blood culture STH	*Klebsiella oxytoca*	97.92	2a	3a, 2b
Blood culture STH	*Klebsiella pneumoniae*	767.98	2b	3a, 2b
Blood culture STH	*Klebsiella pneumoniae*	851.98	2b	3a, 3b
Blood culture STH	*Klebsiella pneumoniae*	842.98	2b	3a, 3b

Table 1. Continued.

Origin	Species	Laboratory Code	Hybridization	
			Strong	Weak
Blood culture STH	*Enterobacter cloacae*	770.98	3a	2b, 3b
Blood culture STH	*Enterobacter cloacae*	814.98	3a	2b, 3b
Blood culture STH	*Enterobacter cloacae*	810.98	3a	2b, 3b
Blood culture STH	*Enterobacter aerogenes*	743.98	2b	3a, 3b
382010	*Citrobacter freundii*	382010	2b, 3b	3a
Blood culture STH	*Proteus mirabilis*	827.98	1a, 1b	
Blood culture STH	*Proteus mirabilis*	838.98	1a, 1b	
Blood culture STH	*Proteus mirabilis*	703.98	1a, 1b	
Blood culture STH	*Serratia marcesens*	1087.98	2a, 2b, 3a, 3b	
Blood culture STH	*Pseudomonas aeruginosa*	37036	4b	
Blood culture STH	*Pseudomonas aeruginosa*	812.98	4b	
Blood culture STH	*Pseudomonas aeruginosa*	728.98	4b	
Blood culture STH	*Pseudomonas aeruginosa*	714.98	4b	
Blood culture STH	*Pseudomonas aeruginosa*	760.98	4b	
Blood culture STH	*Pseudomonas aeruginosa*	702.98	4b	
Blood culture STH	*Pseudomonas aeruginosa*	845.98	4b	
Blood culture STH	*Pseudomonas aeruginosa*	37036	4b	
Blood culture STH	*Stenotrophomonas maltophilia*	822.98	4b	
Blood culture STH	*Stenotrophomonas maltophilia*	824.98	4b	
CF patient LH	*Burkholderia cepacia*	H7	4b	
CF patient LH	*Burkholderia cepacia*	F3	4b	
CF patient LH	*Burkholderia cepacia*	TR1	4b	
CF patient LH	*Burkholderia cepacia*	H9	4b	
Blood culture STH	Coryneform	Co1	No Hybridization	
Blood culture STH	Coryneform	Co2	No Hybridization	
Blood culture STH	*Candida albicans*	C1	No amplicon or Hybridization	
Blood culture STH	*Candida albicans*	C2	No amplicon or Hybridization	

Table 1. Footnote

STH = St. Thomas' Hospital, GH = Guy's Hospital, LH = Lewisham Hospital, CF = Cystic fibrosis. NCTC = National Collection of Type Cultures, VRE = vancomycin resistant enterococci. MR = methicillin resistant, MS = methicillin senstitive

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Form 23/77 : 1.3.00

Agent : Williams Powell & Associates